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Studies on in vitro Neutralization of Adenoviruses

By

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With 9 Figures

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Introduction

The mechanism of the inactivation of animal viruses by antiserum is still poorly understood. Many theories have been advanced and various mathematical expressions, with appropriate constants, have been fitted to the experimental findings. The interpretation has, however, varied with the experimental procedure employed and so far there does not seem to exist any universal theory that can account for all phenomena described by various authors.

In the studies of *Andrewes*¹ and *Sabin*² a reaction in vitro between virus and antibody was doubted. When data accumulated demonstrating that reactions between virus and serum actually occur in vitro the attention was directed towards the nature of the mechanism involved. *Todd*³ and *Andrewes*¹, working with fowl plague and vaccinia virus respectively, showed that an inactive mixture of virus and antibody could be rendered infectious simply by dilution of the mixture. As noted by *Todd*, *Behring* already in 1899 demonstrated a similar phenomenon in toxin-antitoxin reactions⁴. Thus an atoxic mixture of tetanus toxin and antitoxin could be rendered toxic by dilution. The "dilution phenomenon" has since been reproduced with various virus-antibody systems like influenza (*Burnet*⁵, *Taylor*⁶), papilloma virus (*Bryan* and *Beard*⁷) and *Theiler's* virus (*Gard*⁸).

As an interpretation of this dilution phenomenon it has been suggested^{8,9,34} that the virus-antibody reaction actually proceeds in two steps. The primary union is reversible and does not involve a real inactivation of the virus. With an increasing time of contact between virus and anti-

body, however, a dissociation is rendered more difficult which results in a more definite inactivation. The term "immunoinactivation" was recently proposed by *Gard*⁸ for the second step of the neutralization reaction.

The nature of the dilution phenomenon is, however, still under discussion. *Burnet*¹⁰ and *Dulbecco*¹¹ have recently stressed the influence of excess antibody carried over to the test system. The presence of such free antibody in sufficient concentration may interfere with the progress of the infection. The reactivation by dilution may therefore be only apparent and explained by reduction in free antibody concentration in the test system.

One of the most extensive discussions of the neutralization of virus by antibody is still that of *Burnet et al.*¹². The elaboration of the method of "pock counting" on the chorioallantoic membrane of embryonated eggs enabled them to study the neutralization of several animal viruses from a quantitative point of view. Although the authors did not present a definite opinion on the nature of the inactivation process they advanced the hypothesis that the reaction between virus particles and antibody molecules was a reversible adsorptive process. The adsorption of antibody molecules onto a virus particle would diminish the chance of the virus initiating infection in a susceptible cell. Furthermore, the probability of infection would decrease the more antibody molecules that were adsorbed per virus particle. The authors assumed, that, under idealized conditions, the fraction of virus surviving were inversely proportional to the concentration of antibody. If S is the concentration of antibody, V the original concentration of virus and v the remaining concentration of active virus, the relationship would assume the form

$$\log S = \log V - \log v + b,$$

where b is a constant. The slope of this neutralization curve has, however, been shown to vary with the virus-host system studied (for references see: *Tyrrell* and *Horsfall*¹³ and *Tyrrell*¹⁴). The relationship would, therefore, better be expressed as:

$$a \log S = \log V - \log v + b,$$

where a and b are constants. As pointed out by *Tyrrell* and *Horsfall* this equation also implies that the so-called percentage law, previously formulated by *Andrewes* and *Elford*¹⁵ for the neutralization of bacterial viruses, is valid because

$$\log \frac{V}{v} = a \log S - b.$$

A somewhat new approach to the problems of animal virus neutralization was tried by *Dulbecco* and coworkers¹¹. The application for tissue

culture of the "plaque counting" technique as used in phage work enabled these workers to obtain quantitative estimations of virus activity with an accuracy earlier laborious to attain in work with animal viruses. From their data they conclude that the neutralization of poliomyelitis and WEE virus are "one-hit" phenomena, and at least as regards poliovirus, irreversible. One antibody molecule should thus be enough to inactivate one virus particle although a higher number of molecules may be adsorbed. However, even in the presence of excess antibody a fraction of virus apparently resisted the neutralizing effect.

The data obtained with different techniques and various virus-host systems have obviously not provided unequivocal information on the nature of the reactions which render the virus particle inactive. Apparently the results obtained with different techniques tend to emphasize only certain sides of a complex picture. Before the presentation of the present studies on the neutralization of adenoviruses it might therefore be appropriate to consider in some detail advantages and disadvantages of the techniques available with regard to a study of this particular group of viruses.

Most experiments reported on the neutralization of virus by antibody have been performed according to one of the classical methods, where varying dilutions of virus are tested against a fixed dose of serum, or varying dilutions of serum are tested against a fixed dose of virus. Antibody activity is expressed in the former method in terms of the neutralization index, in the latter method as the serum titer, i. e. the highest serum dilution that inhibits obvious signs of virus activity. As mentioned above there exists a linear relationship between the logarithm of the dose of virus added and the logarithm of the serum dilution end point. The slope of this "neutralization curve" varies with the host-virus system used for the tests. The position of the curve will to certain extent be dependent upon the period of contact between virus and antibody as well as on the temperature at which the mixtures are kept during this interval.

With the fixed virus-varying serum dose method certain difficulties are, however, unavoidable in systems containing slow-growing viruses, such as adenoviruses. On account of the exceptionally long incubation periods readings for virus titrations are, for practical purposes, often taken after an abbreviated incubation, usually at about 6 days, before true end points have been reached. Small differences in the rates of growth of the virus have profound effects on the titers obtained and, thus, on the dosage of virus applied in the test, the latter in turn affecting the serum titer. Some of these difficulties may be avoided if the virus dose used is based upon the true end point titer. This alternative does; however, present other difficulties. The test has to be run for longer periods of time, which calls for tissue cultures of good condition during

corresponding periods. Furthermore, as the outcome of a neutralization test is usually the result of two effects: the initial neutralization and the secondary inhibition by excess antibody incorporated in the culture, unavoidable changes of medium introduce new problems. It thus appears that the fixed virus-varying serum dose method, although applicable and reliable for ordinary routine work, is not particularly suited for studies of the neutralization of adenoviruses from a more basic point of view.

For quantitative purposes the plaque counting technique of *Dulbecco* and cowork.¹¹ should be most suitable, although not free from disadvantages. Due to the limited number of plaques permitted for accurate counting some mixtures of virus and serum have to be considerably diluted before plating which might result in a varying degree of "dilution effect". On the other hand, with high concentrations of antibody, when dilution is not necessary, a continuing reaction between virus and antibody during the adsorption period cannot be avoided even with this method. Unfortunately, the plaque counting method is not applicable for all viruses and has so far not been adapted to the study of adenoviruses. One obstacle is the long incubation period needed with these slow-growing viruses.

Still other techniques have been employed in studies of virus neutralization. The mixture of virus and antibody may, following an appropriate contact period, be titrated for residual virus activity. This method was used by *Gard*⁸ in his studies on immunoinactivation of *Theiler's* virus and has also been found to be of great value for demonstrations of low concentrations of poliovirus antibody¹⁶. As limiting dilutions of adenovirus require long incubation to cause cytopathic changes and as the incubation period, as will be shown, is further prolonged when virus is mixed with antibody, it is difficult always to establish the true end points due to spontaneous degeneration of the tissue cultures.

In the present experiments the so-called incubation time titration method was used. The application of such methods for estimation of virus activity was first described by *Bryan* and *Beard*¹⁷ who worked with rabbit papilloma virus and by *Gard*¹⁸ in his studies on *Theiler's* virus. As shown by these authors^{7,8} the technique may also be used with advantage in studies of some problems concerning the neutralization of viruses. The basis for its application to the present study was the observation¹⁹ that the time required for appearance of cytopathic changes was a linear function of log concentration of seed virus.

Provided the conditions are carefully standardized the incubation time, therefore, offers an accurate, although relative measure of virus activity. It should be noted, however, that the virus dose-incubation time curve may vary both with the virus and the host cell system employed.

The neutralization experiments to be described were mainly performed with adenovirus type 3. Virus was left in contact with antiserum for

varying periods of time after which residual virus activity was determined by end point titrations. The incubation periods were recorded for each separate dilution. It will be shown that the dose-incubation period relationship of virus thus treated deviated from the linearity demonstrable in control titrations performed on mixtures of virus with normal or heterologous serum. The cause of this deviation is studied and discussed.

Material and methods

Tissue cultures: Roller tube cultures of HeLa cells containing about 10^5 cells and 1.5 ml of medium per tube were used for all titration purposes. Stationary cultures, in Roux flasks, of the same cells were employed for production of the virus materials used in the experiments. The preparation and maintenance of the cultures have been given in detail elsewhere¹⁹. The medium used after inoculation consisted of bovine amniotic fluid (BAF) with the addition of 0.5 per cent Bacto-tryptose²⁰, 100 IU penicillin and 100 μ g streptomycin per ml. In a few experiments the epithelium-like cell strain Detroit 6 (*Stulberg* and cowork.²¹) has been used. These cells were handled according to the technique adopted for the HeLa strain, with the exception that the fluid medium was changed about twice as often.

Viruses: Most of the experiments have been performed with passages of a strain of adenovirus type 3 (B 1304) isolated during a study²² of an epidemic of pharyngoconjunctival fever. Virus pools No. 2181, 2660 and 2803 were from the second and third passages of this strain. The harvested fluids were centrifuged at 3500 r. p. m. for 30 minutes (pool 2181) or 2 times for 1 hour (pool 2660 and 2803). The supernatants were distributed in glass-sealed ampoules and stored in a CO₂ cabinet until used. Similar preparations of three other virus strains were used in a few experiments. These included the prototype 3 strain "G. B."²³ (kindly provided by Dr. *Huebner*) and the strain "Sutherland"²⁴ (kindly supplied by Dr. *Neva*) which also belongs to type 3, as well as the type 5 strain 4574/54 (pool 1726¹⁹).

Sera: Human sera: Acute phase and convalescent sera were collected during the epidemic of pharyngoconjunctival fever mentioned above²². Paired sera from three patients were used in the present study. They were selected because the acute phase specimen showed no evidence of neutralizing activity in a screening test.

Hyperimmune sera: These were prepared in rabbits as follows: Serum 1490 was drawn from a rabbit immunized against the prototype 3 strain. The animal received three weekly injections, 5 ml intravenously and 5 ml intramuscularly, of tissue culture fluid containing about 10^7 ID₅₀/ml. Nine months later three weekly injections were again given, each time with 5 ml intravenously. One week after the last injection the rabbit was bled by heart puncture.

Serum 2659: 5 ml amounts of the virus pool 2181 (type 3) were injected once a week, altogether 6 times, into the hind leg of a rabbit. The animal was bled by heart puncture immediately before each injection. The sera thus collected are recorded as serum 2659 1 week, 2 weeks, . . . 6 weeks, respectively.

Before the start of the course of immunization a normal serum was obtained from each rabbit.

All sera were heated at 56° for 30 minutes. They were stored frozen at about — 25° C until used.

Virus titrations: The method employed for determination of the relationship between virus concentration and time for cytopathic changes has been described previously¹⁹.* The 50 per cent end point, based on the final readings, was computed according to *Kärber*²⁵.

Neutralization tests: Only a few serum dilution titrations are included in the present work. Serial dilutions* of serum were mixed with a fixed virus amount (for details see below). Each mixture was tested in 5 roller tubes, the inoculum being 0.2 ml per tube. The 50 per cent serum dilution end point was computed according to *Kärber*²⁵.

The incubation time titration method employed in most of the present experiments may be described as follows. Equal volumes of serum and virus were mixed. 0.2 ml amounts of serial tenfold dilutions* of the mixtures were inoculated into roller tube cultures of HeLa cells. Variations of concentrations, temperature and time factors are recorded in the separate experiments. Five tubes were used per dilution. The cultures were inspected for cytopathic effects at about the same time each day for, if possible, three weeks.

Results from several titrations of control mixtures of virus and normal rabbit serum or BAF were used for computing a standard regression line¹⁹. Similar titrations were performed with mixtures of various immune sera and viruses. In the latter cases the mean of the incubation periods was computed for each virus concentration causing cytopathic changes in all cultures inoculated.

In a few experiments dilution of the virus-serum mixture at inoculation into the culture tubes was avoided by addition of the culture medium to the serum and virus components before mixing. The mixture was then added as fluid medium to empty culture tubes.

Definitions: In this paper the time of contact between virus and antibody before inoculation will be named "contact period", whereas "incubation time" always refers to the interval between inoculation and the first appearance of typical cytopathic changes.

* Phosphate-buffered saline pH 7.1–7.2 was, as previously¹⁹, used as diluent.

Experiments and results

Serum dilution end point determination

As an example of serum dilution end point determinations one such experiment will be briefly described. Rabbit hyperimmune sera 2659 1 week and 5 weeks, respectively, were used. Serial twofold dilutions of the sera were mixed with a fixed dose ($1/_{100}$) of type 3 virus 2803. The virus-serum mixtures were kept at room temperature for a few minutes before inoculation into the cultures. Table 1 shows the serum dilution end points recorded at various intervals after inoculation as well as the corresponding readings of the virus control titration. The figures illustrate clearly the slow progress of the cytopathogenic effect in the virus titration and the gradual "break-through" in the serum-virus mixtures in successively higher serum dilutions as incubation is extended. It is evident that true end points are not reached even after 18 days of incubation.

Table 1. Serum dilution titers as determined after various periods of inoculation

Time after inoculation days	Virus control 50 per cent end points on the time indicated	Log serum titer	
		Serum 1 week	Serum 5 weeks
7	0.5	≥ 2.4	≥ 3.0
9	1.5	2.14	2.65
11	2.5	1.53	2.25
15	4.0	0.75	1.65
18	≥ 4.5	< 0.6	0.8
21	≥ 4.5		< 0.6

By plotting on a log/log scale the successive apparent serum titers against the corresponding apparent virus titers "neutralization curves" are obtained, representing for both sera approximately straight lines with a slope of about 2. According to *Ginsberg*²⁶ type 3 virus should characteristically give a conventional neutralization curve with a slope around 1. As *Ginsberg* varied the actual amounts of both virus and serum but used a fixed incubation time the difference in techniques excludes a direct comparison of the results. It should be recalled, however, that, according to *Dulbecco et al.*¹¹, the slope of the neutralization curve will be dependent upon the multiplication rate of the virus used. As shown previously¹⁹ the growth rate varies for different strains and even lines of adenovirus. It seems not unlikely, therefore, that *Ginsberg's* conclusion that the slope of the neutralization curve is characteristic for certain types of adenovirus has only a limited validity.

The results reported in Table 1 emphasize, furthermore, the importance of a carefully standardized technique without which readings taken at a fixed time after inoculation will hardly be reproducible.

Determination of residual virus activity of virus-antibody mixtures

Although the incubation time titration method was mainly employed in the experiments to be presented it might seem worthwhile to consider some data based on titrations of remaining virus activity of virus-antibody mixtures. In Table 2 some 50 per cent end point titers of mixtures of type 3 virus and human convalescent or rabbit immune sera are recorded.

Table 2. 50 per cent end points of titrations performed with mixtures of virus and various sera

Material	50 per cent end point titer (log)				The readings taken after inoculation (days)
	Time of contact of virus-serum before titration; in hours				
	0	1	3	6	
Equal volumes of virus pool 2181 + acute phase serum B1286	6.75	7.5	7.0	7.5	21
Equal volumes of virus pool 2181 + convalescent serum B 1691	7.0*	6.1*	5.7*	4.9	21
Equal volumes of virus pool 2181 + convalescent serum B 1646	6.3	5.5	5.5	—	16
Equal volumes of virus pool 2181 + rabbit serum 1490 undiluted	2.9	2.7	—	2.7	16
diluted 10 ⁻¹	4.5	3.25		3.5	
diluted 10 ⁻²	7.1	6.5		6.5	
Normal serum 10 ⁻¹				6.7	
Normal serum 10 ⁻²				7.1	
Equal volumes of virus pool 2660 + rabbit serum 2659					14
1 week	4.8	4.7	—	4.1	
4 week	4.0	2.3		2.1	
Normal serum undiluted				6.5	14

* = mean of two titrations.

In this experiments virus was mixed in equal amounts with the serum used. After varying periods of contact serial tenfold dilutions of the mixtures were inoculated into cultures and the residual virus activity determined in terms of 50 per cent end points. The results of simultaneously performed control titrations of mixtures of virus and acute phase or normal rabbit serum are included. The virus activity apparently decreased with the time of contact of virus and antibody in the primary

mixture. However, the data also illustrate some disadvantages of the end point titration method. Thus they give no conclusive evidence regarding the optimal period of contact between virus and antibody needed to reach a presumptive "steady state". The differences between the 1 hour and the 6 hour values, where they exist, may not be significant. Due to the condition of the tissue cultures incubation could only be followed for limited periods, varying from one experiment to the next. Under such conditions it is obviously doubtful to what extent true end points are reached.

It appeared that the application of the incubation time titration method might yield more detailed and precise information. For each separate dilution step in the titration series the mean incubation period obtained was, therefore, recorded and compared with the standard curve, obtained from titrations of virus alone or mixed with normal serum. The results are reported below.

Incubation periods obtained with mixtures of virus and normal sera

In a previous paper¹⁹ it was demonstrated that the addition of tryptose to the tissue culture medium increased the degeneration rate in cultures inoculated with one of the adenovirus strains. Before interpretation of the neutralization tests it was therefore important to find out if the enrichment of the medium with serum had any similar influence upon the virus dose-incubation time relationship.

1. *Human sera*: In Table 3 some data from 18 titrations of a type 3 virus pool (2181) are recorded. They were all run as controls in connection with neutralization tests. Nine of them were performed on mixtures of virus and the acute phase sera from three human beings whose second blood specimens were used in the neutralization tests to be described. The regression coefficients of the individual experiments did not deviate more from each other than could be expected from chance alone ($P > 0.2$). Furthermore, contact between acute phase serum and virus at 37° C for 3 hours did not seem to inactivate any measurable quantity of the virus used. The regression coefficients of the combined data ("within" the experiments) were estimated at 1.875 (x on y) and 0.509 (y on x), respectively. The high value (0.974) of the correlation coefficient indicated a linear relationship. With time chosen as the independent variable the variance of virus dilution was estimated by the mean square residual (σ^2) after elimination of the variation that refers to the regression. A value of 0.12 was obtained. The regression line of these combined data is used as standard curve in Fig. 2.

2. *Normal rabbit sera*: A control with the corresponding preimmunization serum was included in all neutralization tests where rabbit immune

Table 3. Titrations of virus pool 2181

Materials	Nr. of experiment	Contact period of virus and BAF or serum before titration, in hours	b	r	$\bar{x}$	$\bar{y}$	50 per cent end point titers (14 day readings)
Mixture of virus and BAF	2401	0	1.727	0.972	3.75	6.53	7.1
		1	1.976	0.981	3.36	7.00	6.5
		3	1.821	0.980	3.37	6.66	6.5
	2381	0	1.788	0.974	3.5	6.63	6.5
		1	1.937	0.978	3.26	6.30	7.1
		3	1.840	0.982	3.00	5.68	6.3
	2359	0	1.975	0.967	2.91	5.75	6.0
		1	2.00	0.979	2.92	5.83	6.7
		3	1.880	0.996	3.41	6.89	6.5
Mixtures of virus and acute phase sera from three human beings	2401	0	1.834	0.988	3.5	6.43	6.5
		1	1.864	0.972	3.46	6.67	6.9
		3	1.936	0.980	3.41	6.66	6.7
	2381	0	1.860	0.975	3.00	5.92	6.5
		1	1.813	0.985	2.83	5.22	6.5
		3	1.860	0.973	3.00	5.88	6.5
	2359	0	1.900	0.968	3.00	5.76	6.3
		1	2.040	0.967	3.00	5.83	6.1
		3	1.863	0.984	3.33	6.07	7.1

b = regression coefficient. r = correlation coefficient.

sera were studied. Titrations of mixtures of virus and these particular normal sera indicated no influence of the presence of the serum, even if the mixture had been kept at 37° C for 6 hours and then at 4° C over night. Regression lines computed from the combined data of those titrations where the virus was mixed with either normal rabbit sera or with BAF are included as reference lines in Fig. 3, 5-6, 8 and 9. Data for the reference lines, computed for the two virus batches employed are tabulated in Table 4. It should be mentioned in this connection that titrations performed with mixtures of adenovirus type 3 and rabbit hyperimmune sera against type 5 and 7 came out as those performed with normal rabbit sera. Immune sera obtained after immunization with virus cultured in HeLa cells seemed thus not to have such an effect on the cell system that it influenced the normal virus dose-incubation time relationship.

On account of its importance in cross neutralization tests between different types of adenoviruses it should, however, be mentioned that some normal rabbit sera have been found to influence the virus dose-incubation time relationship. Fig. 1 illustrates the results of a titration of an adenovirus type 5 mixed with such a "normal" serum. The mixture

Table 4. Data from titrations of virus pools 2181 and 2660

Material	b (x on y)	b (y on x)	r	σ^2_x	$\bar{x}$	$\bar{y}$	Mean of 50 per cent end point titers (reading after 14 days)
Virus pool 2181 (5 titrations, 4 in combination with normal rabbit serum)	1.722	0.556	0.976	0.13	3.42	6.47	6.8
Virus pool 2660 (14 titrations, 9 in combination with normal rabbit serum)	1.862	0.511	0.970	0.14	3.31	6.27	6.8

b = regression coefficient. r = correlation coefficient.
 y = incubation period. x = neg. log of virus dilution.

was kept at 37° C for 1 hour before being titrated. The resulting regression line shows a steeper slope and a prolongation of the incubation time before cytopathic changes, averaging 7.7 days against 5.2 days in the controls. Similar results have been obtained with another normal rabbit serum. They resemble those obtained with mixtures of virus and low concentrations of hyperimmune serum as illustrated below. Contact of the animals with adenovirus before hyperimmunization cannot be excluded. Hyperimmune sera from the rabbits mentioned were not used in any of the experiments reported in this paper.

Incubation periods obtained with mixtures of virus and immune sera

1. *Human convalescent sera*: The virus dose-incubation time relationships in titrations of mixtures of virus and a human convalescent serum

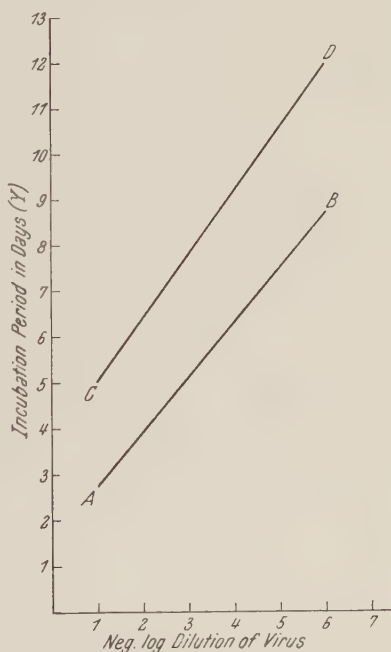


Fig. 1. Regression lines calculated from titrations of virus pool 1726. A—B = control titration ($b = 1.20$). C—D = titration of mixture of virus and a "normal" rabbit serum ($b = 1.41$).

are illustrated in Fig. 2. With the possible exception of the mixture with the longest time of contact before dilution, the dose-incubation

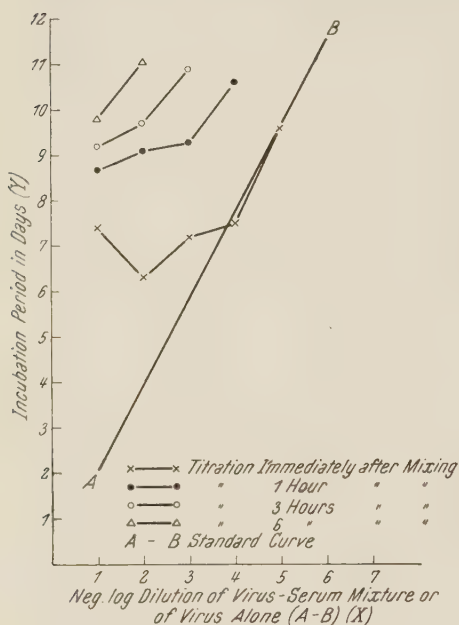


Fig. 2. Curves obtained from titrations of mixtures of virus (2181) and convalescent serum (1691).

time relationship clearly deviated from linearity. Even the titration performed immediately after mixing virus and serum showed for the lowest dilution tested, i.e. 10^{-1} , a considerable prolongation of the incubation period indicating a reduction in virus activity. Upon dilution, however, this reduction gradually decreased until at high dilution levels the values did not differ from those of the control. Dilution thus resulted in an apparent reactivation of the mixture. With increasing time of contact between virus and antibody before titration the increasing incubation periods indicated a progressive loss of virus activity, whereas the "dilution effect" became less pronounced and there was a

gradual approach to a linear dose-incubation time relationship.

In a normal titration, the dose-incubation time curve would be expected to run a linear course with a slope parallel to the reference line. The degree of deviation from this course can be used as a measure of

Table 5. Estimation of the dilution effect in the experiments illustrated in Fig. 2

Dilution	Effect of dilution performed	
	Immediately	After 1 hour of contact
10^{-1}	k	k_1
10^{-2}	$k + 1.6$	$k_1 + 0.8$
10^{-3}	$k + 2.2$	$k_1 + 1.7$
10^{-4}	$k + 3.0$	$k_1 + 2.0$
10^{-5}	$k + 2.9$	

k and k_1 = dilution effect of the first 10 fold dilution. (Undiluted mixtures were not tested. Hence the effect of the first step of the tenfold dilutions cannot be numerically evaluated.)

the "dilution effect". By interpolation in the standard curve this deviation in days can be translated to a corresponding apparent reactivation expressed in log virus units. The values of Table 5 have been calculated in this fashion and correspond to the "immediate" and "1 hour" curves of Fig. 2. It may be seen that the "dilution effect" of the titration performed immediately after mixing continues to increase over four 10-fold dilutions.

The difference in 50 per cent end point values between the control titration and the titration of the virus-serum mixture performed after 6 hours at 37° C amounted to 2.3 log. However, if the same difference between the virus titers was estimated by the incubation time method (described previously) it was found to be about 4 log. Apparently the incubation period obtained in these titrations of virus-serum mixtures was longer than expected and did not correspond to the value for the surviving virus fraction as obtained by the 50 per cent end point method.

The results of titrations of mixtures of virus with two other convalescent sera came out similarly.

2. Rabbit hyperimmune sera:

The results of titrations of type 3 virus (pool 2181) after mixture with different concentrations of rabbit hyperimmune serum 1490 (undiluted, diluted 10⁻¹ and 10⁻²) are graphically illustrated in Fig. 3. The outcome of titrations of the mixture of virus and a 10⁻¹ dilution of serum was essentially similar to that described above for a mixture of virus and a human convalescent serum. It should be noted, however, that the last part of the curves showed a steeper slope than that of the standard line. With undiluted serum there was, after a remarkably long incubation time, a negative slope only, even if the mixture was kept for 6 hours at 37° C before titration. The titrations of a

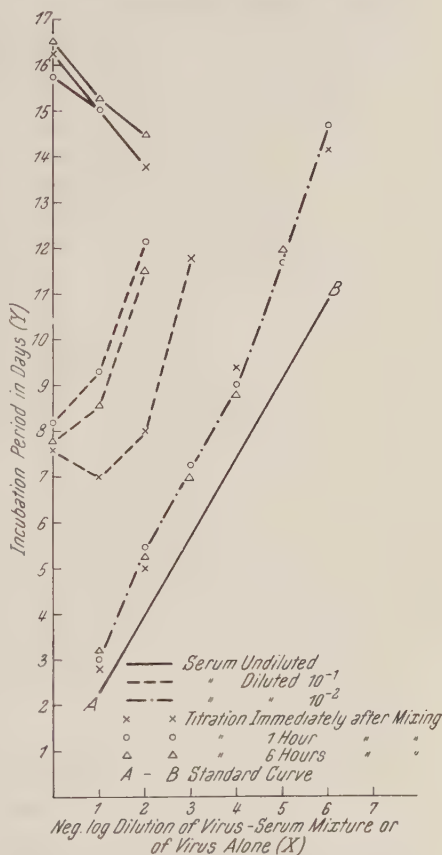


Fig. 3. Curves obtained from titrations of mixtures of virus (2181) and various concentrations of rabbit immune serum (1490).

mixture of virus with an equal volume of a 10^{-2} dilution of serum showed an approximately linear dose-incubation time relationship. The slope was, however, significantly ($P < 0.001$) steeper than that of the control. The difference in incubation time thus amounted to about three days in the highest dilutions. In ordinary virus titrations this would correspond to a difference in virus activity of about 1.5 log.

To find out if the contact between virus and serum had permanently changed the virus to a slower multiplication rate harvests from cultures

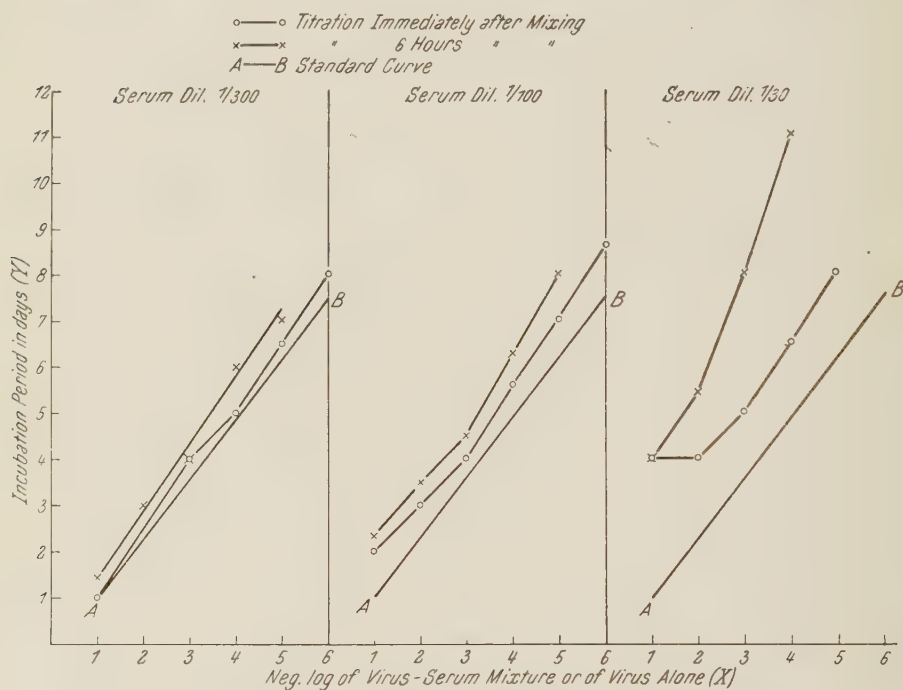


Fig. 4. Curves obtained from titrations of mixtures of virus (2803) and various concentrations of rabbit immune serum (1490).

with delayed incubation periods were tested and found to have regression lines identical to that of the original virus.

In summary, the titrations of mixtures of virus and antiserum deviated from the dose-incubation period relationship obtained with the virus controls. With high concentrations of antibody in the primary mixture there was a negative slope only. Lower antibody doses caused an upward bending of the first part of the curve followed by a slope steeper than that of the control. Diluted serum, finally, caused a steeper slope only.

The observation that the regression lines obtained with mixtures of virus and diluted hyperimmune serum came out steeper than those of the control titrations seems noteworthy. Some experiments were carried out in order to confirm this phenomenon. In these experiments samples of another virus batch (pool 2803) were mixed with dilutions of the same rabbit hyperimmune serum (1490) and tested on HeLa cell cultures and, in addition, also on another cell system, Detroit 6. The virus used showed, in the Detroit 6 cell system, a linear dose-incubation time relationship with a regression coefficient of 1.3. In the HeLa cell system the same virus batch showed a slower degeneration rate with a regression coefficient of 2.2. From Fig. 4 which illustrates the experiments employing the Detroit 6 cell system, it is evident that the neutralization phenomena mentioned above were reproducible. The part of the line that was not directly influenced by relatively high concentrations of antibody (see below) again showed a steeper regression compared to the controls. Furthermore, with increasing doses of antibody as well as with increasing time of contact between virus and antibody before inoculation the slope increased. The experiments in the HeLa cell system performed simultaneously came out similarly.

In the above experiments the serum of a thoroughly immunized rabbit was used throughout. In other experiments an attempt was made to find out whether serum specimens drawn at various levels of immunization would differ principally from each other. In order to avoid every possible "dilution effect" by inoculation, 1.5 ml volumes of the reaction mixtures were inoculated into empty cultures.

Accordingly, mixtures were prepared consisting of a 10^{-1} dilution in BAF of type 3 virus (pool 2660) and a similar 10^{-1} dilution, in BAF, of

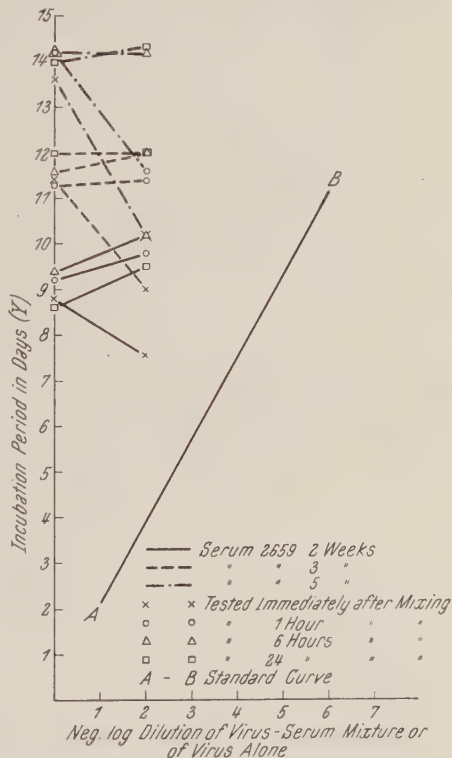


Fig. 5. Incubation periods of mixtures of virus (2660) and rabbit immune sera (2659; 2 weeks, 3 weeks and 5 weeks). After various time of contact between the reagents the mixtures were inoculated undiluted as well as diluted 10^{-2} .

serum 2659 drawn after, 2, 3 or 5 weeks. Inoculation was done immediately after mixing as well as after contact periods at 37° C of 1 hour and 6 hours, and 6 hours at 37° C followed by about 18 hours at 4° C. At the same intervals a 10⁻² dilution of the mixture was made and similarly inoculated.

The results of these experiments are recorded in Fig. 5. The incubation periods obtained for the original mixtures (i. e. without dilution after mixing) did not vary significantly between samples taken immediately after the virus and serum were mixed and those taken subsequently to 1, 6 or 24 hours of contact between the reagents. Remarkable differences in incubation time were, however, observed between those samples where the original mixtures were diluted 10⁻² at various intervals after mixing. In Table 6 the "dilution effect" obtained for the different sera is recorded. Apparently the dilution effect reached a minimum following a contact period of at least 1 hour except for the mixture containing the 5 week serum where the minimum was still not reached after this interval.

Table 6. Dilution effect computed from experiments given in Fig. 5

Contact period virus-antibody before dilution	0	1 hour	6 hours	24 hours
Serum 2 weeks:	2.6	1.7	1.5	1.5
„ 3 „	3.3	1.9	1.7	2.0
„ 5 „	3.9	3.5	2.0	1.8

The outcome is well in accordance with the results, described above, of full titrations performed with mixtures of virus and serum 1490.

Influence on the incubation period by variation of the proportions of serum and virus: A couple of experiments were designed to investigate the effect of variation of the proportions of virus and antibody. From Fig. 6 it is seen that the 10⁻² dilutions of the two sera (2659, 1 week and 4 weeks) had no discernable effect on undiluted virus although they had been in contact with the virus at 37° C for 6 hours before inoculation into the cultures. The same concentrations of antibody did, however, appreciably prolong the incubation period if they were mixed with ten times less virus and, even more, with a hundred times less virus. Both sera disclosed the same trend although they came out at different levels. It might be mentioned that the 50 per cent neutralization end point of serum 2659, 1 week, against 100 TCD₅₀ of virus was only 1/32.

Influence of free antibody remaining in the culture medium: In the above experiments the medium of the tissue cultures was not changed until one week after inoculation. During the first week a continuous influence of reactive antibody could thus be expected at least in the

lowest dilutions where enough antibody might be available. Some experiments were devoted to this problem.

A series of tenfold dilutions of virus was inoculated into cultures. Three hours later corresponding dilutions of immune serum were added to the tubes in 0.1 ml amounts. In another titration series 0.1 ml amounts of the same serum diluted 10^{-1} was added to each culture, irrespective of the virus dilution. The results are illustrated in Fig. 7. Immune serum

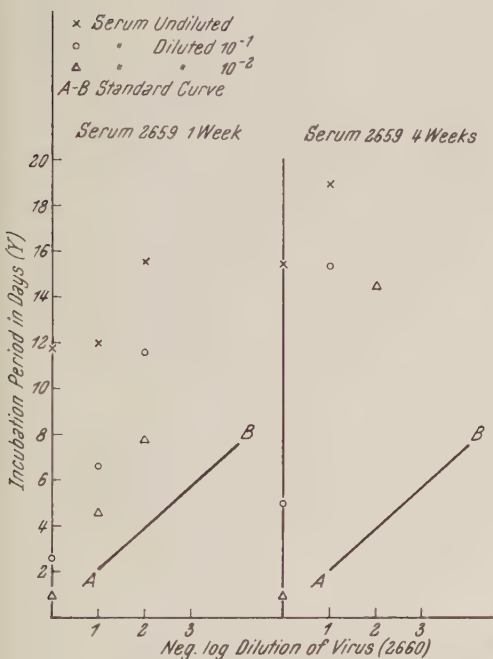


Fig. 6. Incubation periods obtained for separately prepared mixtures of virus and serum. The mixtures were kept at 37°C . For 6 hours + at 4°C over night before inoculation.

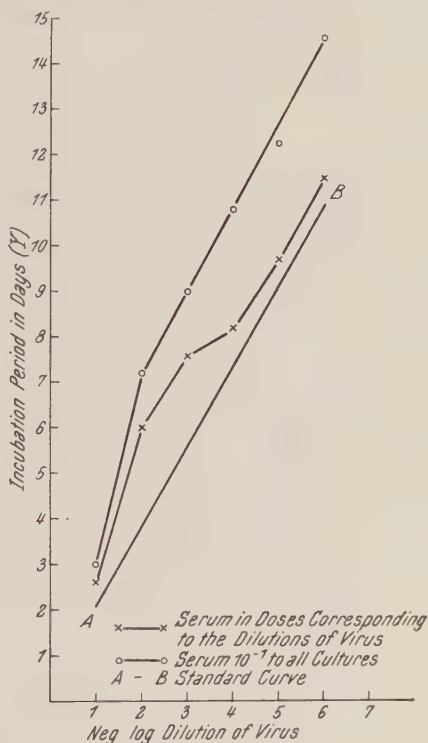


Fig. 7. Curves obtained from titrations of virus (2660). Serum (2659, 3 weeks) was added to the cultures 3 hours after inoculation.

in sufficient concentration had obviously a delaying effect on the virus activity. From the 7th day on when the medium was changed without replenishing the serum content this effect disappeared. It is obvious, however, that the phenomena observed in titrations of virus-antibody mixtures were not reproduced by addition of antibody after adsorption of virus to the host cells.

In order to prevent a further interaction between carried over reactive antibody and newly released virus in the titration experiments with

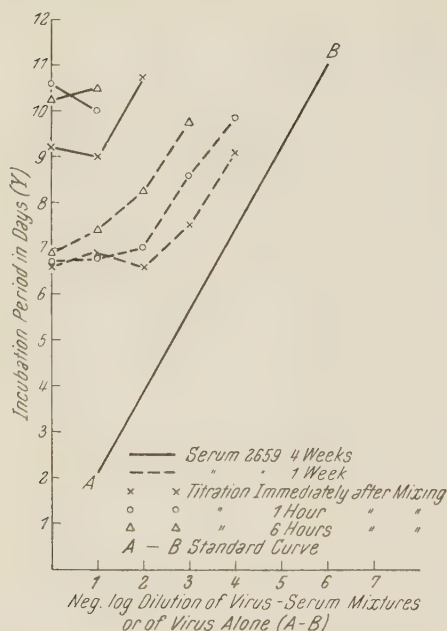


Fig. 8. Curves obtained from titrations of mixtures of virus (2660) and serum (2659 1 week and 4 weeks). All cultures were washed 3 hours after inoculation.

mixtures of virus and serum, the culture tubes were in some experiments removed from the roller drum three hours after inoculation and washed by three changes of 2 ml PBS before the addition of fresh medium. A virus control titration similarly washed three hours after inoculation showed no significant drop in 50 per cent end point titer nor any prolongation of the incubation periods.

In Fig. 8 results of titrations of type 3 virus (pool 2660) mixed with an equal volume of rabbit immune serum 2659, 1 week or 4 weeks respectively, are recorded. These cultures were all washed three hours after inoculation. Results from titrations of virus and serum 2659,

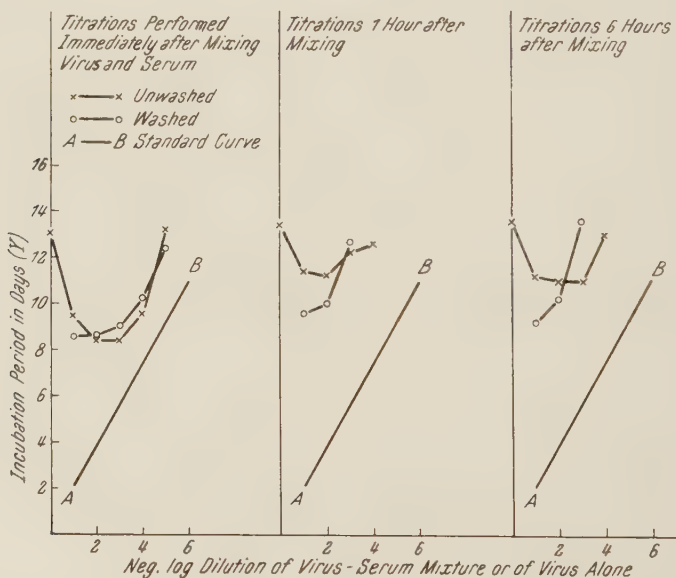


Fig. 9. Curves obtained from titrations of mixtures of virus (2660) and serum (2659 3 weeks). Washed and unwashed cultures are compared.

Table 7. Dilution effect of titrations illustrated in Fig. 9

The values are calculated with the 10^{-1} dilution as zero value

Dilution	Dilution effect of titrations performed					
	Immediately		After 1 hour		After 6 hours	
	Unwashed	Washed	Unwashed	Washed	Unwashed	Washed
10^{-1}	—	—	—	—	—	—
10^{-2}	1.4	1.0	1.1	0.7	1.1	0.5
10^{-3}	2.4	1.7	1.5	0.2	2.1	— 0.4
10^{-4}	2.9	2.1	2.2		1.9	
10^{-5}	1.9	1.9				

3 weeks, are shown in Fig. 9 where washed and unwashed cultures are compared. Apparently carried over free antibody did influence the results of titration experiments as the incubation periods were shortened following washing of those cultures which were inoculated with the lowest dilutions of the virus-antibody mixtures. This effect of washing was not very apparent in the titrations performed immediately after mixing as can be seen from the values of the dilution effect recorded in Table 7. The remaining deviations from straight lines, which were quite significant, can hardly be explained by any presence of reactive antibody.

Table 8. Difference in incubation period between two mixtures of virus 2660 and serum 2659 4 weeks

One of them was prepared by mixing rather concentrated reagents and subsequently diluting the mixture, the other by mixing diluted reagents. All dilutions were prepared in BAF and the specimens inoculated in 1.5 ml amounts into empty culture tubes.

	Final dilutions kept at 37° C before inoculation: hours	Incubation periods: days
Mixture of 1/10 virus + 1/10 serum . Mixture diluted 1/1000:		
Immediately after mixing	0	10.6
“ “ “ “	1	11.4
“ “ “ “	6	13.5
After 1 hour at 37° C	0	16
Mixture of 1/10000 virus + 1/10000 serum ..	0	8.2
	1	8.6
	6	9.8
Control: estimated from the standard curve, virus 1/10000 without serum	0	7.3

It may, furthermore, be noted that the previously mentioned phenomenon of a steeper slope in the high dilution range was more evident in the washed cultures of this experiment. In Table 7 it manifests itself as a decreasing "dilution effect".

Influence of the initial concentration of virus and antibody on the extent of neutralization: In Table 8 the neutralization (as revealed by prolongation of the incubation period) following dilution of a mixture of concentrated reagents is compared with that obtained after mixing diluted components. Obviously, contact of virus and antibody in a more concentrated state, although only for a short moment, resulted in a longer incubation period before the appearance of cytopathic changes. The neutralization was not reversed if the dilution of the concentrated mixture was kept at 37° C before inoculation; on the contrary it increased similarly to that of the mixture of diluted reagents. It did not reach the level obtained by a prolongation of the contact period before dilution, however.

Availability of reactive antibody in virus-antibody mixtures: In the above neutralization experiments the interest has mainly been focused on the effect on the virus. The experiment illustrated in Table 9 was designed to test for the availability of reactive antibody in a virus-anti-serum mixture after various periods of contact between the reagents. If fresh virus was added to the reaction mixture immediately after this was made up the extent of the neutralization was apparently about the same as if all the virus and antibody had been mixed at once. The small discrepancy in the figures might be explained by the fact that actually a

Table 9. Incubation periods obtained by adding fresh virus to a virus-antibody mixture after various contact periods. The Sutherland virus strain, and serum 2659, 5 weeks were used in the experiment

	Final dilutions kept at 37° C before inoculations: hours	Incubation periods: days
Mixture of 1/10 virus + 1/10 serum	0	13
Inoc. 0.1 ml amount	1	13.8
	6	14.5
Mixture of 1/10 virus + 1/10 serum Equal volume of fresh virus 1/20 added:		
Immediately after mixing.....	1	13.3
After 1 hour at 37° C.....	1	11.3
After 6 hours at 37° C.....	1	11.0
Inoc. 0.2 ml amounts		
Control estimated from the standard curve; 0.1 ml virus 1/20, no serum..		2.4

double amount of virus was present in the latter mixture. With an increased period of contact between the antibody and the first aliquot of virus the amount of reactive antibody available for neutralization of new virus decreased, as revealed by shorter incubation periods. There were, however, still considerable amounts of reactive antibody left.

In a parallel experiment the influence of the presence of host cells during the contact of virus with antibody was tested. 1.5 ml amounts of the primary virus-serum mixtures were, immediately after mixing, inoculated into empty roller tube cultures of HeLa cells. The fluid of one tube was harvested after 1 hour in the roller drum at 36–37° C. Other cultures were harvested after 6 hours, 24 hours and 1 week, respectively. Immediately after harvest, the fluids were tested for remaining virus activity and remaining reactive antibody by tests performed as those illustrated in Table 9. The presence of host cells during the contact of virus with antibody influenced the results given in the table in the following way: During the first hour some virus was apparently adsorbed to the cells (incubation period 14.6 days compared to the 13.8 days of Table 9). However, the virus-antibody reaction seemed to progress to about the same extent (incubation period after addition of fresh virus 11.8 days compared to the 11.3 days of the table). With increasing periods of contact of virus with antibody and host cells the virus activity progressively decreased (incubation periods 16 days, 17 days and > 3 weeks after 6 hours, 24 hours and 1 week, respectively). However, the reactive antibody remained at about the same level (incubation periods 11.2, 11.4 and 11.3 days).

Discussion

The above experiments mainly concern the phenomena observed in titrations of mixtures of adenovirus type 3 with antibody. It was demonstrated that in titrations of mixtures of virus and antibody the virus dose-incubation time relationship deviated from the straight line found in ordinary virus titrations¹⁹ in two respects:

I. A flatter slope (or even a negative one) within the low dilution range of the titration, provided the antibody concentration of the primary mixture was high enough.

II. A steeper slope within the high dilution range.

Ad I. The first steps of dilution of a mixture containing high concentrations of both reagents resulted in incubation periods shorter than those obtained with undiluted mixtures (as manifested by the negative slope of the first part of the curve). With an increasing period of contact between virus and antibody before inoculation this "dilution effect" decreased. If the amount of antibody in the primary mixture was reduced, the appearance of the titration curve was gradually "normalized".

Some of the excessive prolongation of the incubation period in those cultures inoculated with the highest concentrations of virus-hyperimmune serum mixtures could be eliminated by washing the cultures 3 hours after inoculation. It was, furthermore, shown that, without washing, reactive antibody remained in the medium at an appreciable level up to the first change of medium. These observations indicate that the deviation of the titration curve, was to a large extent the result of a continuing effect of reactive antibody in the cultures. The importance of such an effect of remaining antibody has earlier been stressed by *Burnet*¹⁰ for in vivo neutralization systems. Experimental evidence of the existence of the effect in vitro was recently presented by *Dulbecco et al.*¹¹ in their study of WEE virus and poliovirus with the plaque count technique. They also stressed the importance of this effect of remaining antibody for the occurrence of the commonly observed "dilution phenomenon".

However, a considerable abnormality of that section of the curve, which represented cultures inoculated with high concentrations of virus and antibody remained, even if the cultures were carefully washed. It should be recalled that an adsorption period of three hours after inoculation was allowed for, however. During this time obviously some progress of the virus antibody reaction should be expected. It is well known that the fraction of virus neutralized within a certain time increases with the concentration of the serum (^{11, 27, 28}, present experiments). It seems probable, therefore, that a progressive virus-antibody reaction during the adsorption phase accounts for some of the bending of the curve.

The fact that a dilution of a virus-antibody mixture often results in an increased virus activity, has been attributed to a reactivation of already neutralized virus^{1, 3, 5-8, 27}. Whether or not a reactivation of virus-antibody complexes upon dilution also contributed to the "dilution phenomenon" observed in the present experiments can hardly be definitely decided upon from the data available. In agreement with *Dulbecco's* experience with poliovirus¹¹ we found no reactivation after prolonged incubation of a dilution of a virus-hyperimmune serum mixture. Even if the dilution was performed immediately after mixing, a procedure which results in a marked "dilution phenomenon", the neutralization reaction continued to progress after dilution. If anything, this result would speak against a reactivation.

Ad II. At present no easily conceivable explanation can be offered for the steeper slope regularly found within the high dilution range in titrations of virus mixed with hyperimmune serum or throughout the whole titration if the antibody concentration of the primary mixture was low. Some possibilities will, however, be briefly discussed. It has previously been demonstrated¹⁹ that, in virus titrations, there exists a linear relationship between incubation period (i. e. time after inoculation before

the appearance of cytopathic changes in the tissue cultures) and log concentration of adenovirus. This relationship would correspond to an exponential virus growth as shown by the following reasoning. On the assumption that the number of cells stays constant during the experiment, the time T required for total degeneration of the culture can be calculated from the following formula:

$$b^c = n b^{\frac{T}{g}}. \quad (1)$$

Where

b = average burst size, i. e. number of infectious units yielded per cell.

c = number of cells in the culture.

n = number of primarily infected cells.

g = average generation time per cycle.

From (1) we obtain

$$T = g + \frac{g}{\log b} \cdot \log \left(\frac{c}{n} \right). \quad (2)$$

The number of primarily infected cells, n , is a function of the number of infective virus units in the inoculum (v). Provided that one active virus particle is sufficient to incite infection, i. e. that infection is a one-hit phenomenon, the relationship between n , c , and v will assume the form of the well-known Poisson formula

$$\frac{n}{c} = 1 - e^{-\frac{v}{c}}, \quad (3)$$

$\frac{v}{c}$ = number of infective units per cell = multiplicity of infection (m).

For multiplicities of 0.1 or less $\frac{n}{c}$ is, with a very close approximation, equal to $\frac{v}{c}$; for multiplicities above 0.1 $\frac{n}{c}$ will increasingly lag behind when m is growing. Therefore, it is to be expected that T will be a linear function of $\log v$ over the major part of the range of titration, a deviation from linearity being demonstrable only in the region of very large inocula, which actually agrees well with the observed data.

On the basis of this reasoning an increase in the slope of the incubation time curve can have three different explanations: (a) a change in the ratio $\frac{g}{\log b}$, i. e. a longer generation time, smaller bursts, or both, (b) a variation in the length of the first cycle of virus multiplication, (c) a change in the relation between n and v [equation (3)].

Ad (a). As previously shown¹⁹ the environmental conditions (effect of tryptose) may influence the cell reactions to infection. It was important, therefore, to examine both the possibly favourable effect of normal serum

and the possibly unfavourable effect of anti-cell antibodies in immune serum. Neither one of these factors was demonstrable, however. Therefore, if the effect is to be explained according to alternative (a) it should be through an action of antibody upon the virus. It might be assumed that combination with subneutralizing amounts of antibody would produce a "genetic" change of the particle towards less activity or else that the effect of neutralization would be a selection of slow reproducing, genetically stable virus variants. This possibility, by itself rather unlikely, was ruled out by the observation that the progeny from cultures with retarded multiplication rates behaved exactly as the original virus. More likely is the possibility that antibody has a certain selective effect, combining preferably with the more "avid" or active particles in an inherently inhomogeneous virus population. Excess antibody remaining in the culture would then successively "weed out" the avid virus also in the later stages of virus growth and keep the rate of multiplication below the average without interfering with the genetic properties of the virus. This effect would be more pronounced the higher the antibody concentration. It would, thus, lead to a decrease in the slope of the curve, as actually observed in the region of high antibody concentration. It could hardly account for the consistent increase in the range of high dilutions, however.

It would seem, therefore, that alternative (a) can be ruled out.

Ad (b). It is well conceivable that a combination of antibody with "non-essential" sites in the virus particle might have an effect upon the rate of adsorption of the virus and/or the rate of multiplication in the first cycle. In order to find in such an assumption an explanation of the phenomenon observed one must obviously also assume that the average length (or less likely the average burst-size) of the first cycle is itself a linear function of $\log v$.

The rate of adsorption of adenoviruses has not been studied. Data concerning other virus-cell systems are available, however^{29, 30}. Generally the uptake of virus per unit of time seems to be directly proportional to collision frequency, or when the cell population is kept constant to the concentration of free virus. Most probably the same will hold true for adenoviruses. In the present situation one should regard the mixture of virus and antibody as a mixture of variants with different specific adsorption rates. Theoretically it can be shown that the average specific adsorption rate of such a mixture will vary with the proportions of the components whereas the absolute concentration will be without importance as long as the proportions remain unchanged. The alternative (b) would, therefore, lead to a parallel displacement of the regression line. This assumption, thus, does not seem to explain the steeper slope.

Ad (c). There remains the alternative of a change in the relation between n and v . This would imply that the classical Poisson formula would

no longer apply to virus exposed to antibody. An interpretation of this phenomenon cannot be offered at present. Superficially the effect resembles a multiplicity reactivation.

Whatever the mechanism behind the increased slope, the phenomenon indicates that antibody may influence a virus in other ways than by completely inactivating it. This finding seems incompatible with the theory of *Dulbecco* and coworkers,¹¹ according to which the combination of any one antigenic site with a neutralizing antibody should lead to neutralization of the virus particle. On the other hand, the phenomenon may well correspond to the partial effect of antibody on phages described by *Andrewes* et al.³¹ and *Burnet* et al.³². Mixtures of phage and weak anti-serum thus sometimes caused plaques smaller than those seen in the virus controls.

From the practical point of view it seems evident that the incubation time method may be used with advantage for screening of the antibody content of sera as well as for comparison of the antibody content of different sera³³. The chief advantage should be a great saving of labor and cultures. However, the following facts should be kept in mind. On the one hand it is obvious that if the antibody contents of two strong sera are to be compared the sera should either be diluted before being mixed with virus or the virus dose should be selected high enough to permit a "break through" within a reasonable time even after mixture with the largest dose of antibody. On the other hand small amounts of antibody as well as differences within the low concentration range can only be detected if the virus dose is reduced. With such precautions the incubation time method is very sensitive, however.

Besides the precautions mentioned above the influence of the dilution of the virus-antibody mixture with the tissue culture medium should be considered. Variations in the outcome should thus be expected if there are variations in the amount of tissue culture medium or in the contact period between virus and serum before inoculation.

Summary

The incubation time titration method has been applied to a study of the neutralization of adenovirus by antibody. Following dilution of mixtures of virus and serum a marked "reactivation phenomenon" was apparent. The results indicate that this phenomenon was partly caused by remaining reactive antibody in the medium, partly by a faster and more extensive reaction between virus and serum in higher concentrations. Whether or not a real reactivation also contributed to the "dilution effect" cannot be definitely decided upon.

The results indicated, furthermore, that antibody may influence the virus in another way than by a complete inactivation. This effect was

correlated to the dose of inoculum. Some possibilities regarding the mechanism of the phenomenon are discussed.

With suitable concentrations of virus the incubation time titration method has been found applicable for routine work.

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